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MUTAGEN ISOLATION METHODS: FRACTIONATION OF RESIDUE ORGANICS FROM AQUEOUS ENVIRONMENTAL SAMPLES

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16. ABSTRACT

A basic assessment of the mutagenic activity of organic residues from water entails application of the Salmonella test to both the parent residue mixture and to major subfractions obtained by reverse phase and/or normal phase HPLC. Such analyses overcome the presence in particular residue mixtures of components which are antagonistic or toxic for the bioassay. When applied to residues obtained by various procedures, the method reveals a wide range of mutagenic activity among environmental waters, depending upon the source of the water sample, the apparent types of natural and anthropogenic compounds it contains, and the water processing and disinfection procedures used in its treatment. Biologically this range of activity encompasses mutagenic components which are differentiated on the basis of tester strain specificity and the effects of the absence or presence of microsomal activation. Chemically the various mutagenic components are separable by HPLC across the entire range of polar, mid-polar, relatively non-polar and non-polar compounds. Through combinations of specific mutagenic bioassays plus sequential analytical and preparative HPLC fractionations, this method has been shown to be applicable for the isolation of mutagens from residues of a variety of environmental waters.

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INTRODUCTION

The presence of trace levels of anthropogenic organic compounds in environmental waters has heightened concern as to the possible human health impact of chronic exposure to these contaminants via routes such as drinking water. One response to this concern in the United States has been the enactment of a series of Federal Acts, including the Safe Drinking Water Act of 1974, PL 93-523, and the Clean Water Act of 1977, PL 95-217. Although the implementation of this federal legislation has yielded improvements in the nation's drinking water and national waterways regarding substances such as toxic metals (e.g. Council on Environmental Quality 1982), a recent review (Crump and Guess, 1980) concluded that "further studies of the identities, carcinogenicity, mutagenicity, mode of formation and practical methods of removal are needed for the organic contaminants". The goal of our studies has been to understand the biohazardous components of complex residue mixtures of organics from aqueous samples in terms of their prevalence, concentration, chemical structure, origin and mechanism of biological effects. One specific focus of this research effort has been the isolation, identification and chemical/biological characterization of mutagenic components from aqueous environmental samples.

The first step required for this process was the application of methods for the isolation of residue organics from aqueous samples, e.g. drinking water, ground water, wastewater, etc. (Tabor and Loper 1984), using columns of XAD resins to concentrate the organics. The residue organics then can be tested for mutagenicity and other biological end points via a variety of short-term bioassay procedures (Hoffman 1982), and/or fractionated for the isolation of mutagenic components for chemical/biological characterization.

The need for the fractionation of residue organics isolated from water samples to assess mutagenicity and to identify the biohazardous constituents has been discussed (Loper 1980a,b). Based on this need, a biological approach to the chemical fractionation and separation of residue organics is the method of choice for the isolation of biohazardous compounds from complex mixtures of residue organics. We introduced a general method based upon high performance liquid chromatography (HPLC) for the fractionation of residue organics from drinking water that featured assessment of the distribution of constituents by polarity using an analytical HPLC separation followed by preparative scale HPLC separation of the residue organics for mutagenicity testing and compound identification (Tabor et al. 1980, Tabor and Loper 1980). The strategy for this method is summarized in Figure 1. This approach led to the first identification of a previously unidentified mutagen from drinking water residue organics (Tabor 1983).

The use of HPLC for the separation of residue organics from aqueous samples for mutagenicity testing has been extended to the studies of many other types of water samples from different parts of the world. Baird and co-workers (Baird et al. 1980, Nellor et al. 1984) have utilized such an approach in their studies of mutagenicity of residue organics from drinking water, river water, storm run-off and reclaimed wastewaters, and other wastewaters. Jolley and co-workers (Jolley and Cumming 1979, Cumming et al. 1980) have applied HPLC to the separation of wastewater effluent residue organics to study the relationship of mutagenicity to disinfection methods and to fractionate residue organics from other polluted waters for bioassay of separated fractions. In a study of genotoxic components of oil shale retort process water, Strniste et al. (1983) first separated these samples by a classical liquid/liquid extraction scheme followed by HPLC separa-

tion of the extracts for bioassays. One Dutch research group (Kool et al. 1984) has reported in these proceedings the use of HPLC to fractionate drinking water residue organics for mutagenicity assessment. Our research group has made extensive use of HPLC for the fractionation of drinking water and wastewater residue organics (citations above and Loper et al. 1983, 1984a, Tabor et al. 1984). In these studies, residue organics from samples of ground- and river water and from samples of various stages in the processing of both drinking waters and of diverse waste waters have been fractionated. This approach has provided for a better assessment of the mutagenic potential of such residue organics. The purpose of this report is to present the specifics of our HPLC approach as applied to residue organics from a variety of aqueous samples, particularly as the method is used in the isolation of mutagenic constituents for compound identification. Examples of the separation of residue organics from a variety of aqueous environmental samples will be used to illustrate the applicability of the approach.

METHODS

Instrumentation

High performance liquid chromatography (HPLC) separations were performed on a Waters Associates (Milford, MA) system consisting of two Model 590 pumps, a Model U6K injector, a Model 680 automated gradient controller, and both Model 440 fixed wavelength, 254 nm, and Model 480 variable wavelength absorbance detectors. Chromatograms were displayed on a Fisher Recordall Series 5000 two-pen recorder. For preparative scale (mg level) separations, a Waters Associates Z-module® radial compression column (RCM) unit was utilized. The RCM unit was fitted with an 8 mm by 10 cm column packed with 10 um silica particles bonded

with octadecylsilane for reverse phase separations; for normal phase separations, similar RCM columns packed with 10 um silica were used. For analytical scale (ug level) separations, prepacked 3.9 mm by 30 cm columns (Waters Associates), packed with 10 um silica particles bonded with octadecylsilane for reverse phase separations or with 10 um silica for normal phase separations, were used.

Mass spectrometry (MS) analyses were performed on a Kratos MS 80 (Manchester, U.K.) high resolution mass spectrometer as described previously (Tabor 1983, Tabor and Loper 1984).

Residue Organics and Other Chemicals

Residue organics were isolated from various aqueous samples via our XAD chromatographic procedure (Tabor and Loper 1984, Tabor et al. 1984). Finished drinking water I (DWI) was from a city that draws its raw water from a river impacted by chemicals from numerous industrial, municipal and agricultural sources. To prepare finished drinking water, the raw water is treated by a series of settling, coagulation and floculation steps, with the final product being chlorinated to a residual level of 1 to 2 mg/L. Finished drinking water II (DWII) was from a city that draws its raw water from a network of streams and rivers that principally drain wilderness regions. The raw water is settled in a series of reservoirs, following which it is chlorinated to a residual level of 2 to 4 mg/L for distribution. Finished drinking water III (DWIII) was from a city that draws its raw water from a major U.S. aquifer system. The raw ground water is treated by pH adjustment with lime to remove minerals, followed by a series of settling, coagulation and floculation steps. The final product is chlorinated to a residual of 1 to 2 mg/L, a process which readjusts the pH to

the neutrality range. Since this aquifer is recharged in part by stream bank infiltration from a river subject to multiple points of contamination from industrial, municipal and agricultural sources, residue organics were isolated from both the river water (RW) and the raw ground water (GW) for mutagenesis/separation assessment. In addition to these drinking waters and raw waters, both influent wastewater (IWW) and effluent wastewater (EWW) from a municipal sewage treatment plant, heavily (>80% volume) impacted by industrial discharges, were examined.

Organic solvents for HPLC separations, methylene chloride, methanol, isopropanol, hexane and acetonitrile, were obtained as HPLC grade from Fisher Scientific (Cincinnati, OH). Type I water (USEPA 1979) for HPLC and for the preparation of other aqueous solutions was purified as described previously (Tabor and Loper 1980). All HPLC solvents were filtered through a 0.45 um Millipore® membrane filter (Millipore Corporation, Bedford, MA) and degassed by 15 min of sonication while under reduced pressure immediately prior to use. Sample enrichment purification cartridges, SEP-PAKS® (Waters Associates), packed with silica particles bonded with octadecylsilane were activated and used according to methods described previously (Tabor et al. 1980, Tabor and Loper 1980). All other chemicals were of reagent grade and were used without further purification.

Biological Analysis

Tester strains TA98 and TA100 for the Salmonella microsomal mutagenicity tests were provided by Dr. B. Ames. Mutagenesis assays were conducted as described (Loper et al. 1984a, Tabor and Loper 1984). Characteristic properties of the bacterial tester strains were verified for each fresh stock, and their

mutagenicity properties were verified again using positive and negative controls as part of each experiment as recommended (Ames et al. 1975). Mutagenesis tests requiring metabolic activation utilized polychlorinated biphenyl mixture Aroclor 1254 induced rat liver 9000 x g supernatant fraction, S9, from Litton Bionetics (Kensington, MD). Mutagenesis assays, without (-S9) and with (+S9) metabolic activation, were conducted as described previously (Loper et al. 1978, Tabor and Loper 1980, 1984a,b). The detection of mutagenic activity in experimental samples was based upon a dose dependent response exceeding the zero dose, spontaneous control value, by at least two fold; i.e. the ratio of total revertant colonies per plate to spontaneous colonies per plate was ≥ 2. In some situations involving HPLC subfractions where the amount of sample was limiting, semiquantitative determinations of mutagenesis were made as described previously (Loper et al. 1983, Tabor and Loper 1984). All recoveries of bioactivity from concentrated or fractionated residue organic samples were based upon an expression of mutagenesis per liter equivalent, representative of the original water sample. Typical mean revertant colony counts, -/+ standard error, obtained from spontaneous plates and positive control plates from our laboratory for the time period of the experiments described herein, were reported recently (Loper et al. 1984a, Tabor and Loper, 1984).

HPLC Separations

Analytical Scale Separations - A preliminary analytical reverse phase HPLC separation of an aliquot of the residue organics was conducted in order to characterize the sample of residue organics according to the polarity of the components. The separation was accomplished by injecting 25 ul of a 1 ug per uL solution of the residue organics [sample dissolved in a minimum volume of aceto-

nitrile, diluted with water until a volume equal to the acetonitrile is added or until the solution becomes cloudy; in the latter case, additional acetonitrile is added, about 10% of the total volume or until the solution becomes clear again] into the HPLC unit operating under the following conditions: flow rate, 2.0 ml/min; initial mobile phase, water. Following sample injection, the column was washed with water for 5 min or until no more 254 nm absorbing components were eluted i.e. no peaks >5% of full scale absorbance setting. After the water wash, a linear mobile phase gradient of 45 min duration was initiated from 100% water to 100% acetonitrile. On completion of the gradient, the column was washed with acetonitrile for 10 min or until no more UV absorbing components were eluted. In general, results from this HPLC separation indicate the composition of the sample of residue organics. For example if the majority, >75%, of the sample components eluted at a solvent composition of 80% water:20% acetonitrile or later, the sample would be ready for separation via preparative scale reverse phase HPLC to collect fractions for mutagenesis testing. However if the majority, >75%, of the sample eluted before the 80% water:20% acetonitrile solvent composition, then the sample would be examined via an analytical scale normal phase HPLC separation prior to scale-up to the preparative level.

To run a preliminary analytical scale normal phase HPLC separation, 25 uL of a 1 ug per uL solution of residue organics [sample dissolved in methylene chloride, minimum volume, then diluted with an equal volume of hexane, using the same procedure and criteria followed for the acetonitrile/water sample solutions above] was injected into the HPLC unit fitted with a normal phase column. The column was washed with hexane, flowing at 2.0 ml/min for 5 min or until no more UV absorbing components were eluted. Following the hexane wash, a linear gradient of 30 min duration was initiated from 100% hexane to 100% methylene

chloride. On completion of the gradient, the column was washed with methylene chloride until no more UV absorbing components were eluted. Following this wash, a linear mobile phase gradient of 30 min duration was initiated from 100% methylene chloride to 100% isopropanol. On completion of this second gradient, the column was washed with isopropanol for 10 min or until no more UV absorbing components were eluted. The results of this HPLC separation identified those samples most suited for preparative scale normal phase separation into sub-fractions for mutagenesis testing.

Initial Preparative Scale HPLC Separations - Based on the results of the analytical scale HPLC separation, one of the following preparative scale HPLC separations was utilized for the initial fractionation of the complex mixture of residue organics for mutagenesis testing. For these separations, mobile phase flow rates of 2.0 ml/min were utilized and sample sizes from 40 to 80 mg were loaded per fractionation run. Each preparative scale run involved the collection of a series of fractions, each of which contained components eluted with a specific combination of isocratic and linear gradient mobile phase compositions. In each case, the respective fraction for each mobile phase composition was collected until no more UV absorbing components were eluted, then the mobile phase composition was changed along with the collection vessel for the collection of the next fraction.

The initial preparative scale reverse phase HPLC fractionation involved the collection of the following five fractions, based on mobile phase composition: Fraction A - 100% water; Fraction B - a 5 min linear gradient from 100% water to 75% water:25% acetonitrile, then isocratic until no more UV absorbing components are eluted; Fraction C - a 5 min linear gradient to 50% water:50% acetonitrile,

then isocratic as before; Fraction D - a 5 min linear gradient to 25% water:75% acetonitrile, then isocratic as before; and Fraction E - a 5 min linear gradient to 100% acetonitrile, then isocratic as before. The fractions were processed for mutagenesis testing and/or further separation according to the following procedures. The aqueous solution fraction, Fraction A, was concentrated via gentle evaporation under a stream of dry nitrogen, according to procedures described previously (Tabor et al. 1984). Fractions B - E were processed via our previously published procedure employing reverse phase SEP-PAK® cartridges (Tabor et al. 1980, Tabor and Loper 1980).

For preparative scale normal phase HPLC separations, the following fractions were collected: Fraction A - isocratic hexane wash until no more UV absorbing components eluted; Fraction B - a 5 min linear gradient from 100% hexane to 65% hexane:35% methylene chloride, then isocratic as before; Fraction C - a 5 min linear gradient from 65% hexane:35% methylene chloride to 35% hexane: 65% methylene chloride, the isocratic as before; Fraction D - a 5 min linear gradient to 100% methylene chloride, then isocratic as before; Fraction E - a 5 min linear gradient from 100% methylene chloride to 65% methylene chloride:35% isopropanol, then isocratic as before; Fraction F - a 5 min linear gradient from the previous solvent to 35% methylene chloride:65% isopropanol, then isocratic as before; and Fraction G - a 5 min linear gradient from the previous solvent to 100% isopropanol, then isocratic as before. These fractions were processed for mutagenesis testing and/or further HPLC separations by gentle evaporation under a stream of nitrogen (Tabor et al. 1984).

Additional Preparative Scale HPLC Separations - After mutagenesis assessment of the HPLC fractions from the initial preparative scale separation above, those

fractions containing mutagenic constituents are further separated on HPLC employing the following strategy. For example if the mutagenic constituents were found to be in Fraction D from an initial reverse phase HPLC preparative scale separation, i.e. a mobile phase composition of 25% water: 75% acetonitrile, a solution of the bioactive fraction would be injected into the HPLC unit. The separation would be accomplished on a reverse phase column, utilizing an initial mobile phase of 40% water: 60% acetonitrile. Following a wash of 5 min or until no more UV absorbing constituents are eluted [collected as Fraction D-1], a linear gradient of 20 min duration to a mobile phase composition of 20% water:80% acetontrile would be run. During this gradient, fractions would be collected at 5 min intervals, i.e. four fractions, Fractions D-2 through D-5. At the completion of the gradient, the collection vessel would be changed for the collection of Fraction D-6 and the column would be washed isocratically with the latter mobile phase until no more UV absorbing constituents eluted or for a time period of 10 min. The six fractions, D-1 through D-6, would be processed, for this hypothetical case, via the SEP-PAK® procedure (Tabor and Loper 1980) for mutagenesis testing. The same general approach would apply for an additional HPLC separation of mutagenic fractions from either initial normal or reverse phase HPLC preparative scale separations.

If further fractionation of mutagenic subfractions is required to isolate constituents from fractions collected during the second separation, a similar HPLC strategy may be employed (Tabor and Loper 1980).

RESULTS AND DISCUSSION

The purpose of this report is to present a general approach for the isolation of mutagenic constituents from residue organics of aqueous environmental

samples. The isolated mutagens then can be characterized chemically as to their identity (e.g. Tabor 1983) or characterized biologically/biochemically as to their mechanism of mutagenesis (e.g. Distlerath et al. 1983, 1984) or other properties. The overall strategy for this approach to isolation is outlined in Figure 1. This strategy of a coupled bioassay/chemical fractionation procedure was proposed by Loper and Lang (1978), based largely upon their mutagenicity and carcinogenicity studies of residue organics isolated by Kopfler et al. (1977) as part of a USEPA 5 city study (Loper et al. 1978) of drinking water organics. Originally the procedure was developed utilizing reverse phase HPLC as a method for the chemical fraction of the complex mixture of residue organics (Loper and Tabor, 1981; Tabor and Loper 1980). However it was found subsequently (Loper et al. 1983) that residue organics from some drinking water samples would require a combination of reverse phase and normal phase HPLC separations to isolate mutagens for compound identification. Therefore, the more general approach described herein was developed.

Mutagenic Activity of Residue Organics from a Variety of Aqueous Samples. Samples of residue organics from differing aqueous environmental sources have been found to contain a wide variation not only in the total amount of mutagenic activity per liter equivalent of water but also in tester strain specificity and requirements for metabolic activation (e.g. Loper 1980a, 1984; Kool et al. 1982). Results of the mutagenic activities for residue organics from a variety of aqueous samples are summarized in Table I. In general, from these data and that reported by others, mutagenic activity of residue organics on a per liter equivalent basis was found to be the greatest for wastewaters from industrially impacted municipal sewage treatment plants (e.g. IWW and EWW, Table I; Tabor et al. 1984, Hopke et al. 1982, 1984) whereas the lowest mutagenic activity appears

to be associated with waters such as raw groundwaters, drinking waters from ground sources (e.g. GW, Table 1; Tabor and Loper 1984), settled river water as a drinking water source sampled prior to disinfection (Loper et al. 1984a), and domestically impacted municipal treatment plant wastewaters prior to disinfection (Loper 1984; Tabor and Loper, unpublished). These data suggest a wide variation in the chemical constituents of the residue organics from different waters. Preliminary analytical scale HPLC separations of the residue organics are required to assess the distribution of organic constituents and are useful in planning preparative scale HPLC separations for mutagenic compound isolation.

Analytical Scale HPLC Separations

Reverse phase HPLC chromatography favors the distribution of the semi- and non-polar constituents of a sample of residue organics, whereas normal phase HPLC chromatography favors the distribution of semi-polar constituents (Tabor 1984). This approach is illustrated in Figure 2 by the chromatograms of residue organics from a wastewater sample separated by both reverse phase and normal phase analytical scale HPLC. These chromatograms show that this sample of residue organics consists of two groups of constituents, i.e. both nonpolar and somewhat polar.

However, the typical HPLC distribution of constituents found in drinking water residue organics is as shown in the chromatogram of a reverse phase HPLC separation in Figure 3. Here the majority of components are eluted in the semi-polar to non-polar range, i.e. with a solvent composition less polar than 60% water:40% acetonitrile. Similar results have been observed for samples of mutagenic residue organics from a variety of drinking waters isolated via XAD chromatography, reverse osmosis or similar methods (Tabor et al. 1980; Tabor and

Loper 1984; Loper et al. 1982, 1984a; Baird et al. 1980; Kool et al. 1984). These results are not unexpected in that Yamasaki and Ames (1977) noted that organic compounds, expected to exert biological effects and capable of passing through biological membranes, are most probably lipophilic compounds of low polarity. The same argument was presented recently by Neal (1983), wherein he added that the toxic components of residue organics from drinking water are likely to be <500 molecular weight. From these arguments, Neal proposed the first priority for compound isolation in order to conduct a more extensive toxicological evalution should be lipid soluble components in this molecular weight range (Neal, 1984).

Preparative Scale HPLC Separations

Results of the analytical scale HPLC separations can be used to develop an approach in the scale-up of the HPLC separations for the preparation of subfractions of the residue organics in quantities suitable for mutagenesis testing and compound isolation. If the analytical scale separation results indicate the constituents of the residues are principally semi- to non-polar, then reverse phase HPLC would be the chromatographic mode of choice to be used for a preparative scale separation. However if the results indicate the constituents of the residues are principally polar to semi-polar, then normal phase HPLC would be the chromatographic mode of choice to be used.

The strategy employed in the first preparative scale HPLC separation, Step I, is to separate the residue organics into discrete groupings of organics according to polarity by using a combination of linear gradient and isocratic solvent elutions. Fractions are collected for each distinct solvent elution employed in the separation. This is illustrated by the preparative scale

separation of residue organics isolated from 120L of drinking water, source DWI, via reverse phase HPLC, Figure 4. Fractions were collected, as indicated, and bioassayed for direct acting mutagenesis. The results of the mutagenesis assays for each fraction, Figure 5, indicate that the majority of the mutagenic components from this sample of residue organics eluted in a relatively non-polar fraction, Fraction 3.

Also in this experiment, proportionate amounts of each fraction were combined then bioassayed. The mutagenic activity for this mixture, mix 1-4 in Figure 5, when compared to the arithimetic sum of bioassay results for the individual fractions collected, Sum 1-4 in Figure 5, indicates the presence of components in the residue organics mixture that are antagonistic to the mutagenic components isolated in each fraction. The results of this experiment illustrate a further application of the preparative scale separation of residue organics: in addition to its use for mutagen isolation, the procedure permits a better assessment of the mutagenicity of residue organics. Such assessments are valuable in cases where mutagenesis testing results for the initial bioassay of the isolated residue organics show toxicity or another form of non-linear dose responses rather than a linear dose response, where the mutagenesis testing results for the initial bioassay show no mutagenic activity. In these cases, the bioassay results could be due to the presence of antagonistic and/or toxic components present in the original mixture of isolated residue organics. The effects of such components have been observed by this HPLC technique for some residue organics isolated from drinking water, e.g. Figure 5, but not all (see Tabor and Loper 1984; Loper et al. 1983). In addition to drinking water residue organics, Alfheim and coworkers (Alfheim et al. 1984, Alfheim and Ramdahl 1984) observed toxicity for residue organics isolated from wood combustion emissions

but were able to accurately assess the mutagenicity of these samples following HPLC fractionation of the residue organics.

Isolation of Mutagens

Applying the general preparative scale HPLC separation procedure outlined in the previous section (Step I), one generally finds the bulk of the mutagenic activity of a given mixture of residue organics to have eluted in one or possibly two of the broad fractional cuts. Therefore, the next and subsequent preparative scale HPLC separations (Step II, III, etc.) of the active fraction(s) from Step I will involve one of two approaches. The first approach utilizes the same chromatographic mode, i.e. reverse phase or normal phase, used in Step I; the second approach involves the alternate chromatographic mode. In either case the general strategy of HPLC fractionation followed by bioassay of an aliquot of the collected fractions is applicable (Figure 1).

The application of the same preparative chromatographic mode for Step II as employed in Step I was described by Tabor and Loper (1980) for the isolation of a TA100 promutagen from drinking water residue organics. The promutagen subsequently was identified as 3-(2-chloroethoxy)-1,2-dichloropropene (Tabor 1983). In the first step, bioactivity was isolated in the 50:50 water:acetonitrile fraction. The Step II HPLC fractionation was accomplished by a shallow linear solvent gradient that started at a solvent composition 5% less in acetonitrile, i.e. the strong solvent, and went to a composition 20% greater in strong solvent than the isocratic solvent composition used for elution of the mutagenic fraction in Step I. [Note: A shallow linear gradient is defined as a 1% solvent composition change per min with a mobile phase flow rate of approximately one column volume per min.] This approach should be generally applicable to the

separation of bioactive fractions from Step I that originally eluted at solvent compositions > 20% strong solvent.

The application of the second approach, use of a chromatographic mode for Step II alternate to that used in Step I, is illustrated by the isolation of a S9 dependent mutagenic subfraction from residue organics isolated from an effluent wastewater sample, EWW in Table I. In this isolation, Step I involved the separation of the residue organics via normal phase preparative HPLC. The resulting chromatogram for this separation is shown in Figure 6. Bioassay of aliquots of the collected fractions showed that >80% of the S9 dependent TA98 mutagenesis had been eluted by 100% methylene chloride in Fraction 5, Figure 6, with no activity for TA98 being detected in the later eluting components. Since this mutagenic fraction eluted in a semi-polar region, preparative scale reverse phase HPLC was applied for the Step II separation. The separation involved a series of isocratic and linear gradient elutions using combinations of water and acetonitrile as shown in Figure 7. Bioassay of the collected fractions showed that >90% of the S9 dependent TA98 mutagenesis had been eluted via an isocratic wash of 50:50 water:acetonitrile into Fraction 4, Figure 7.

Identification of Mutagens

Attempts to further separate this fraction into discrete components via HPLC were unsuccessful, however the sample was found to be suitable for preliminary analysis via mass spectrometry. This procedure showed the sample contained at least three components. The first was found to have a molecular ion at 135.0135 mass units and a characteristic ion at 108.0010 mass units, i.e. loss of HCN. From these and other ions in the spectra, this compound was tentatively identified as benzothiazole. Comparison of this spectra with that obtained on an

authentic sample of benzothiazole confirmed the identification. Although Kinae et al. (1981) have reported benzothiazole to be an S9 dependent mutagen for the Salmonella tester strain, TA1537, our studies indicate this compound does not account for the majority of mutagenic activity in this sample.

A second component in this mutagenic subfraction was shown to have an emperical formula of $C_8H_{10}O_2S$. The compound was tentatively identified as the ethyl ester of benzene sulfinic acid. This structural assignment was made from exact mass analysis and interpretation of the following ions: molecular ion at 170.0400 mass units; a characteristic ion at 141.0029, loss of ethyl; a characteristic ion at 93.9042, loss of phenyl; and other characteristic ions in the spectrum. The mass spectrum of this compound was compared to the spectrum obtained for benzene ethyl sulfone which was run under identical conditions. Although the two spectra were similar, they did not match. Therefore a tentative structural assignment is made at this time with confirmation studies in progress. The possibility of the benzene sulfinic acid ethyl ester being the predominant S9 dependent mutagen in this sample is under investigation.

The third major component detected in this mutagenic sample has not been characterized chemically as of yet. Thus although characterization of this mutagenic subfraction has not been completed two candidates have been identified as possibly contributing to the S9 dependent mutagenesis in this sample.

CONCLUSIONS

A basic assessment of the mutagenic activity of organic residues from water entails application of the Salmonella test to both the parent residue mixture and to major subfractions obtained by reverse phase and/or normal phase HPLC. Such analyses overcome the presence in particular residue mixtures of components which are antagonistic or toxic for the bioassay. When applied to residues obtained by various procedures, the method reveals a wide range of mutagenic activity among environmental waters, depending upon the source of the water sample, the apparent types of natural and anthropogenic compounds it contains, and the water processing and disinfection procedures used in its treatment. Biologically this range of activity emcompasses mutagenic components which are differentiated on the basis of tester strain specificity and the effects of the absence or presence of microsomal activation. Chemically the various mutagenic components are separable by HPLC across the entire range of polar, mid-polar, relatively non-polar and non-polar compounds. Through combinations of specific mutagenic bioassays plus sequential analytical and preparative HPLC fractionations, this method has been shown to be applicable for the isolation of mutagens from residues of a variety of environmental waters.

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LEGENDS TO FIGURES

Figure 1. Schematic of the strategy for the coupled bioassay/analytical fractionation of residue organics isolated from aqueous environmental samples.

Figure 2. Absorbance (254 nm) profile of the analaytical scale HPLC separation of 25 ug of residue organics isolated from an industrially impacted influent wastewater. The separations were accomplished via reverse phase HPLC (top) and normal phase HPLC (bottom). Mobile phases used in these separations were water (H2O), acetonitrile (CH3CN), methylene chloride (MECL), hexane (HX) and isopropanol (IPROH), as indicated.

Figure 3. Absorbance (254 nm) profile of the analytical scale reverse phase HPLC separation of 2.5 L equivalents of residue organics isolated from finished drinking water I.

Figure 4. Absorbance (254 nm) profile of the preparative scale reverse phase HPLC separation of 120 L equivalents of residue organics isoalted from finished drinking water I.

Figure 5. Direct-acting mutagenic activity of HPLC fractions (1-4, Figure 4) obtained by sequential step elution of resisue organics isolated from finished drinking wter I. "MIX" indicates the mutagenic activity measure for an aliquot of residue organics proportionately reconstituted from the separate fractions; "SUM" is the arithmetic sum of the net revertant colonies for each dose for the four fractions.

Figure 6. Absorbance (254 nm) profile of the preparative scale normal phase HPLC separation of 5 L equivalents of residue organics from effluent wastewater I. The bulk of the S9 dependent TA98 mutagenesis was eluted in Fraction 5.

Figure 7. Absorbance (254 nm) profile of the preparative scale reverse phase HPLC separation of the mutagenic Fraction 5, Figure 6. The bulk of the S9 dependent TA98 mutagenesis was eluted in Fraction 4.

COUPLED BIOASSAY/FRACTIONATION PROCEDURE

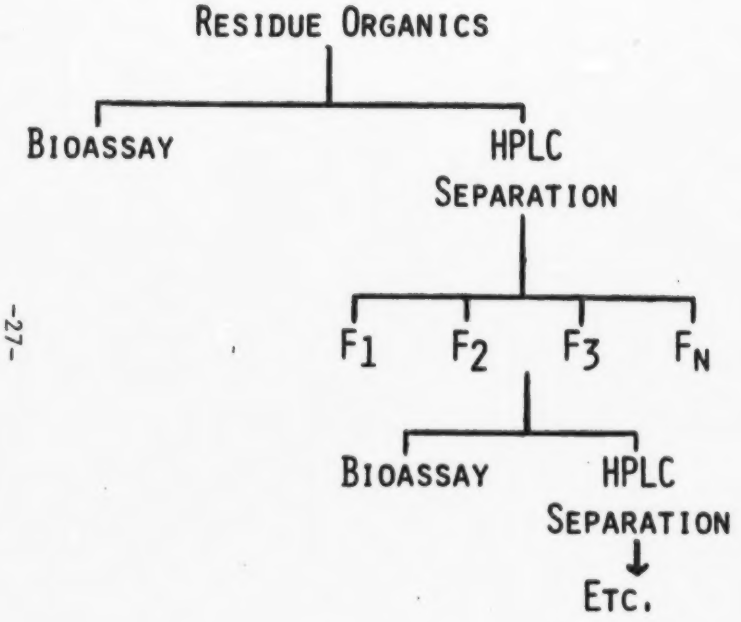

FIGURE 1

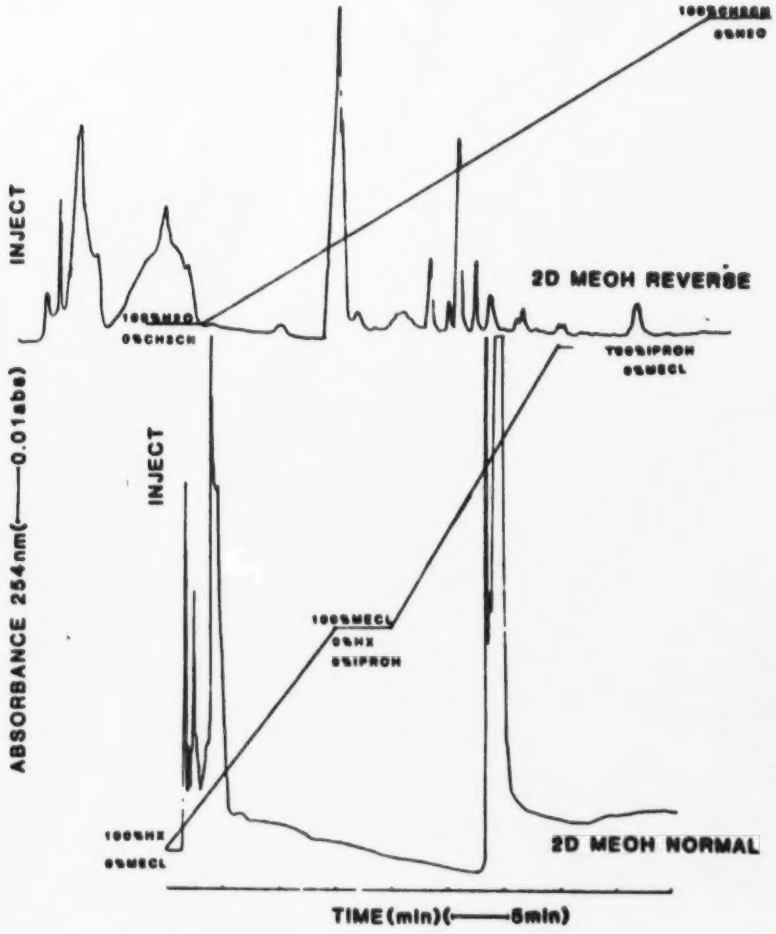

FIGURE 2

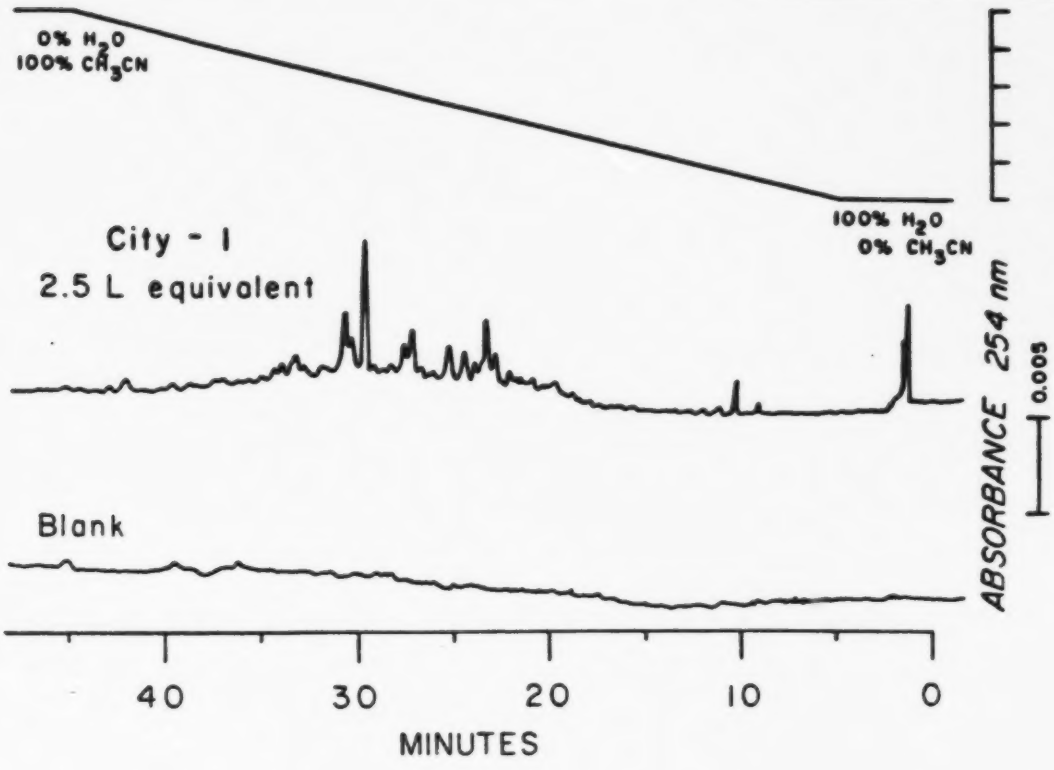

FIGURE 3

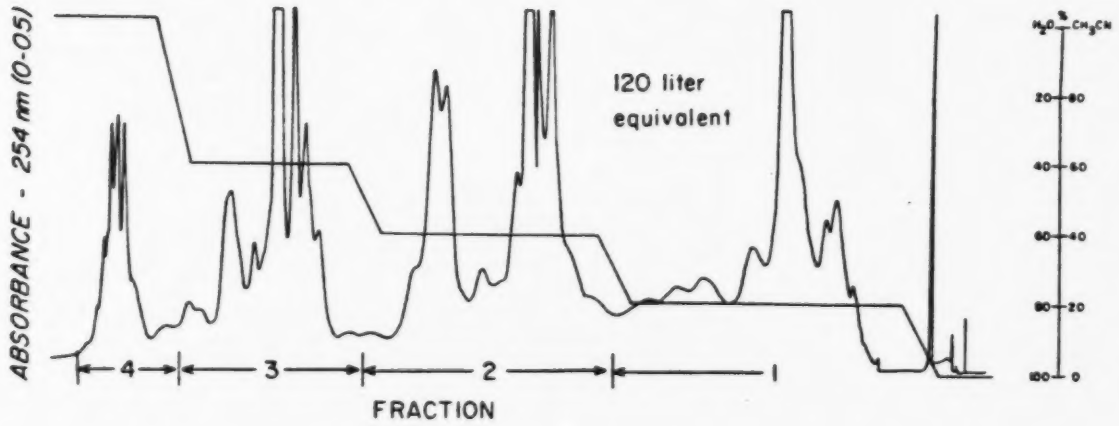

FIGURE 4

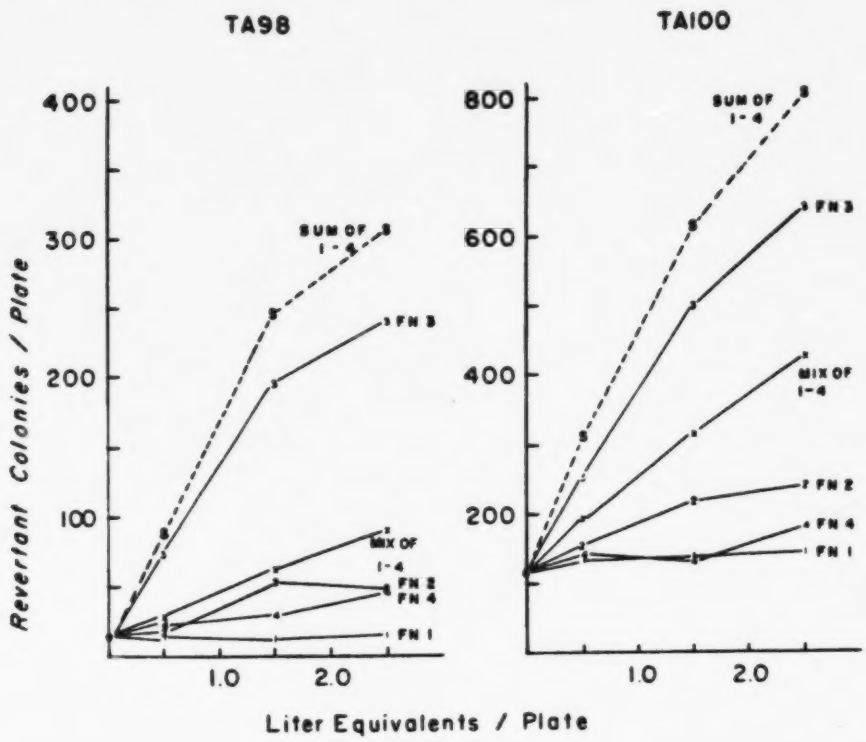
TA98
TA100
400
300
200
100
800
600
400
200
Revertant Colonies / Plate
SUM OF 1-4
FN 3
MIX OF 1-4
FN 2
FN 4
FN 1
1.0
2.0
Liter Equivalents / Plate

FIGURE 5

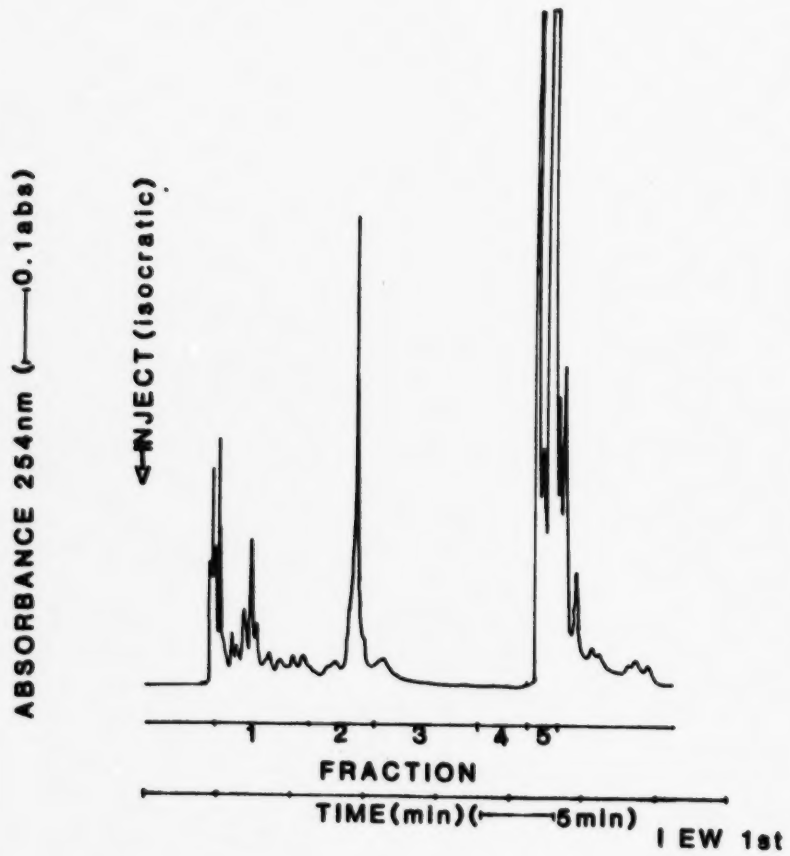

FIGURE 6

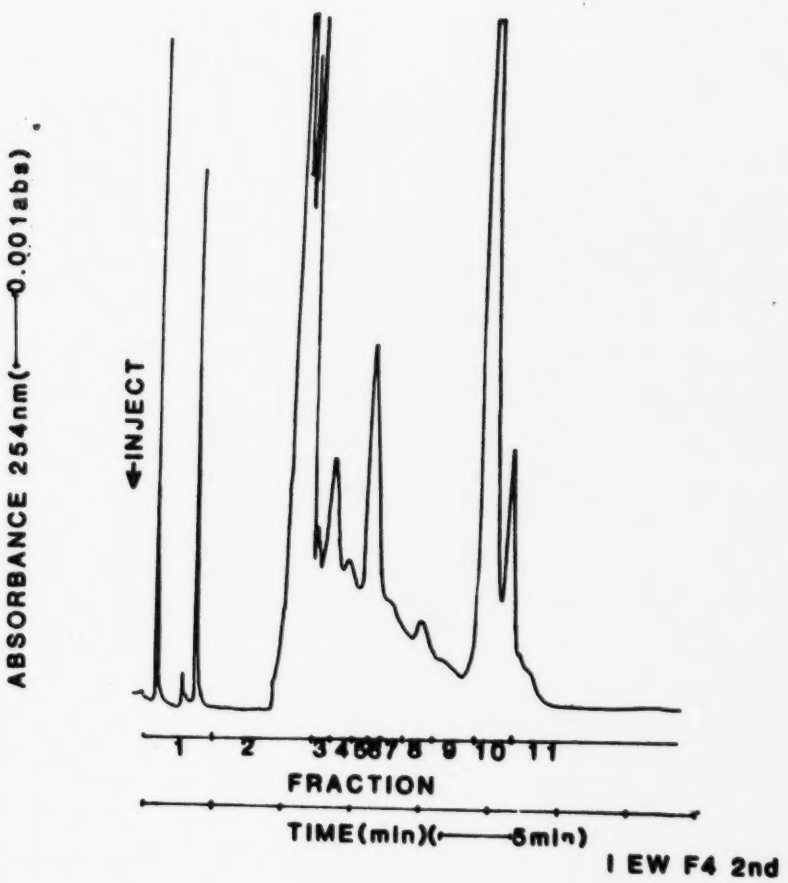

FIGURE 7

TABLE I. Mutagenic Activity for Residue Organics Isolated from a Variety of Aqueous Environmental Samples.

Sample	Net Revertants per Liter-Equivalent*			
	TA98		TA100	
	-S9	+S9	-S9	+S9
Drinking Water I (DW I)	93	36	328	149
Drinking Water II (DW II)	124	48	218	--**
Drinking Water III (DW III)	56	30	184	--
Groundwater (GW)	39	--	--	--
River Water (RW)	53	67	--	--
Influent Wastewater (IWW)	90	12,000	--	--
Effluent Wastewater (EWW)	2,800	3,800	--	--

*Net Revertants = Total Revertants - Spontaneous Revertants, i.e. Controls. Representative Average Spontaneous rates + standard deviation (n>15) over the time period for these experiments were: TA98 - S9, 15 $\pm$ 3; TA98 + S9, 28 $\pm$ 6; TA100 - S9, 122 $\pm$ 10; TA100 + S9, 126 $\pm$ 14.

**Response not equal to nor greater than 2x spontaneous rate.